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Synthesis, Assembly, and Processing of the Env ERVWE1/Syncytin Human Endogenous Retroviral Envelope

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Syncytin is a fusogenic protein involved in the formation of the placental syncytiotrophoblast layer. This protein is encoded by the envelope gene of the ERVWE1 proviral locus belonging to the human endogenous retrovirus W (HERV-W) family. The HERV-W infectious ancestor entered the primate lineage 25 to 40 million years ago. Although the syncytin fusion property has been clearly demonstrated, little is known about this cellular protein maturation process with respect to classical infectious retrovirus envelope proteins. Here we show that the cellular syncytin protein is synthesized as a glycosylated gPr73 precursor cleaved into two mature proteins, a gp50 surface subunit (SU) and a gp24 transmembrane subunit (TM). These SU and TM subunits are found associated as homotrimers. The intracytoplasmic tail is critical to the fusogenic phenotype, although its cleavage requirements seem to have diverged from those of classical retroviral maturation.

The human endogenous retrovirus W (HERV-W) family is derived from an infectious retroviral element which entered the germ line 25 to 40 million years ago (5, 48). The ERVWE1 locus was shown to be the unique copy of the whole family that retained a complete *env* open reading frame (49). We and others have previously demonstrated that this phylogenetically D-type-related HERV-W envelope glycoprotein (Env ERVWE1) (5), also named syncytin, induces in vitro cell-cell fusion (7, 33). This phenotype is also dependent on interactions with the D-type mammalian retrovirus receptor (RDR) (7). Several lines of evidence led us to suggest the physiological role of Env ERVWE1 in placenta formation. Firstly, Env ERVWE1 is preferentially expressed in the human placental syncytiotrophoblast layer (6, 7, 15). Secondly, cellular fusion is abolished by the direct inhibition of Env ERVWE1 expression in primary cytotrophoblastic cells (15). Finally, Env ERVWE1 fusogenic activity has been preserved during hominoid evolution, suggesting that the ERVWE1 *env* gene has been recruited to play a role in placental physiology (32).

The fusogenic form of viral envelope glycoproteins is the outcome of a succession of similar maturation events. The current model is based on structural and functional studies of class I fusion proteins. These proteins include the envelopes of *Paramyxovirus*, *Influenza virus* (hemagglutinin HA_2) (42), *Filovirus*, *Ebola virus* (GP2) (50), *Gammaretrovirus*, *Friend murine leukemia virus* (FrMLV) (p15E), *Deltaretrovirus*, *Human T-cell leukemia virus* (HTLC) (gp21) (25), *Lentivirus*, *Human immunodeficiency virus* type 1 (HIV-1) (51), and *Simian immunodeficiency virus* (gp41). Class I fusion proteins are defined by the following four characteristics: the cleavage of an envelope protein precursor (leading to surface [SU] and transmembrane [TM] subunits derived from the amino and carboxy ends, respectively, of the retroviral precursor), a fusion peptide located just next to the cleavage site, a trimeric complex association, and the ability to form a hairpin structure, also called a coiled coil, in the active fusion conformation. More precisely, fusion proteins are typically synthesized as glycosylated precursors in the lumen of the endoplasmic reticulum (ER) and then modified by the cotranslational addition of N-glycans to the polypeptidic chain and by disulfide bond formation. Next, a trimerization step involving a leucine zipper-like motif, $LX_6LX_6NX_6LX_6L$, occurs. This motif is known to develop strong interactions between oligomers via the formation of a coiled coil structure between three TM (or TM-like) subunits (29, 47). While the oligomers organize as homotrimers (23), they are transported to the Golgi apparatus, where they undergo the last steps of the maturation process, including proteolytic cleavage (4). This cleavage occurs downstream of the last arginine residue of the highly conserved consensus cleavage site K/R-X-K/R-R, recognized by cellular furin-like endoproteases (21, 22), and gives rise to two subunits. In the case of the gammaretrovirus FrMLV envelopes, a disulfide bond is established between the disulfide-isomerase-active CϕϕC motif and the CX_6CC motif, located on the SU and TM subunits, respectively (37). The mature SU-TM trimer can consequently reach the cell surface. During or shortly after budding of the viral particles, the R peptide, consisting of the 16 carboxy-

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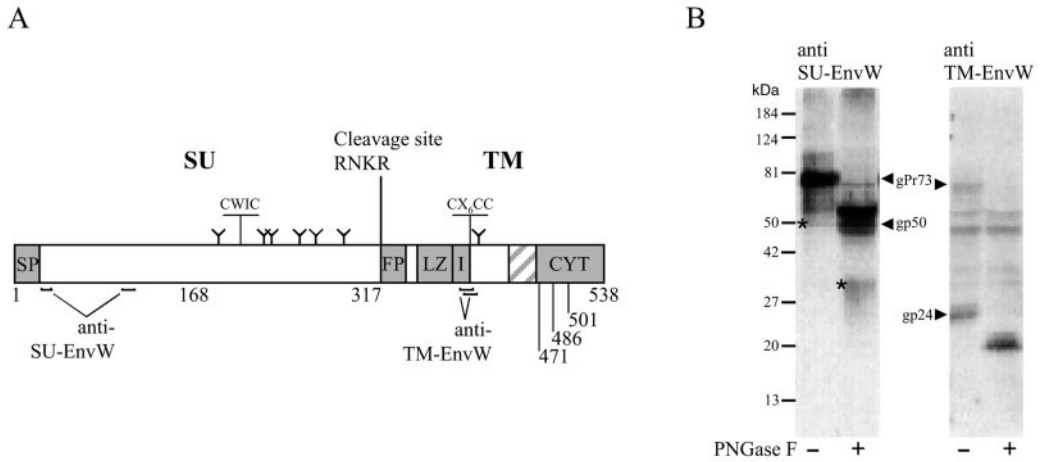

FIG. 1. Organization of Env ERVWE1 and its expression in a human placental model. (A) Characteristic features. The surface (SU, 1-317) and transmembrane (TM, 318-538) domains are indicated. Y, putative N-glycosylation sites (positions 169, 208, 214, 234, 242, 281, and 409) predicted by NetNGly1.0 (http://npsa-pbil.ibcp.fr). Gray boxes indicate the following conserved motifs: SP, signal peptide; FP, fusion peptide; LZ, leucine zipper-like motif; I, immunosuppressive domain; CYT, intracytoplasmic tail (471-538). The hatched box indicates the membrane anchorage domain. Epitope sequences used for immunization are also indicated. (B) Constitutive EnvW expression in human placental trophoblast model. BeWo b30 cells were induced with 50 μM forskolin for 2 days. After induction, the cell lysates were treated with PNGase F (+) or left untreated (−) and then separated by SDS–10% PAGE. Blots were probed with anti-SU-EnvW and anti-TM-EnvW. Arrowheads indicate the positions of gPr73, gp50 SU, and gp24 TM. Asterisks indicate the positions of glycosylated and deglycosylated SU proteins.

terminal residues of the intracytoplasmic tail (19), is cleaved by the viral protease. Altogether, these maturation steps are essential for the acquisition of the envelope protein's fusogenic activity and therefore virion infectivity (40, 41).

The cellular Env ERVWE1 sequence exhibits most of the features of retroviral proteins, as illustrated in Fig. 1A. The characteristic retroviral sequences are as follows, from the amino- to the carboxy-terminal end: (i) a signal peptide, (ii) a CϕϕC motif, (iii) a consensus furin cleavage site, RNKR, that separates the two characteristic glycoprotein domains SU and TM, (iv) a hydrophobic core as a putative fusion peptide, (v) a potential $LX_6LX_6NX_6LX_6L$ leucine zipper motif, (vi) a putative immunosuppressive domain, (vii) a CX_6CC motif, (viii) a hydrophobic stretch of amino acids that are probably involved in glycoprotein plasma membrane anchorage, and (ix) a 69-amino-acid intracytoplasmic domain. The Env ERVWE1 SU and TM domains contain six and one predicted N-glycosylation sites, respectively. In this article, we demonstrate that Env ERVWE1 consists of gp50 SU and gp24 TM subunits, which were efficiently produced in a human placental choriocarcinoma cell line used as a model of the placental trophoblast. Molecular mutant analyses showed that despite 25 to 40 millions years of evolution, the processing of the HERV-W envelope glycoprotein remains essentially similar to that of a classical (exogenous) retroviral protein. Nevertheless, the intracytoplasmic tail, which was demonstrated to be essential for fusion, exhibits an unusual processing mechanism that apparently has diverged from the situation found in infectious retroviruses.

MATERIALS AND METHODS

Cell lines. The following cell lines were used for this study. The TELCeB6 packaging cell line (10), derived from human rhabdomyosarcoma TE671 cells, expresses a nuclear localization signal-*lacZ* reporter MLV vector. HeLa cells (human cervical carcinoma cells) were used as indicator cells in coculture assays. All cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (Gibco) and penicillin-streptomycin at 37°C. BeWo b30 clone cells (human placental choriocarcinoma cell line) (52) were cultured in F-12K nutrient mixture (Kaighn's modification; Invitrogen) supplemented with 10% fetal calf serum and penicillin-streptomycin at 37°C.

Plasmids. DNAs encoding HERV-W envelope glycoproteins were derived by PCR amplification of phCMV-EnvW (clone PH74) (5, 7) and verified by DNA sequencing. The antisense EnvW sequence was inserted into a phCMV-G expression plasmid and used as a control. A furin cleavage site AAAR mutant and a CX_6CA mutant were obtained by use of a Quick-Change mutagenesis kit (Stratagene) to mutate the RNKR motif and the CX_6CC motif in phCMV-EnvW. The truncated envelopes were designated SU (1-317), SU-N (1-168), and SU-C (169-317). Intracytoplasmic tail truncation mutants were obtained from the plasmid phCMV-PH74 after the introduction of stop codons at positions 471 (CYT-1 mutant), 486 (CYT-16 mutant), and 501 (CYT-31 mutant). Expression vectors encoding the amphotropic MLV (A, 1-655) (accession no. AAA46515) and feline endogenous virus (RD114, 1-565) (accession no. CAA61093) envelope proteins and their R-less versions (AR^-, 1-638, and RDR^-, 1-548) were derived from the phCMV-G expression plasmid. Chimeric envelope proteins were designated W/R^+ (W1-473/A623-655), W/R^- (W1-473/A623-638), RD/W (RD1-516/W456-538), and A/W (A1-607/W456-538). Envelope protein transient expression was induced by calcium phosphate precipitation of the different expression plasmids in TELCeB6 cells.

Antibodies. Polyclonal antibodies were raised against the following Env ERVWE1 peptides: residues 21 to 35 and 112 to 129 for rabbit anti-SU (anti-SU-EnvW, used at a dilution of 1:10,000 for Western blot analysis) and residues 390 to 408 and 394 to 413 for rabbit anti-TM (anti-TM-EnvW, used at a dilution of 1:5,000 for Western blotting analysis). The 6A2B2 monoclonal antibody raised against the Env ERVWE1 ectodomain (anti-TM MAb) was previously characterized (7) and used at a dilution of 1:5,000 for Western blot analysis and 1:100 for immunoprecipitation analysis and flow cytometry. An anti-p24 MAb, kindly provided by bioMérieux, was used at a dilution of 1:100 for immunoprecipitation analysis. A monoclonal anti-histidine-tag antibody (anti-His MAb; QIAGEN) was used at a dilution of 1:5,000 for Western blot analysis. A monoclonal anti-actin antibody (anti-actin MAb; Sigma) was used at a dilution of 1:10,000 for Western blot analysis.

Western blot analysis. Transfected cells were lysed in ice-cold phosphate-buffered saline (PBS) containing 0.5% Triton X-100 supplemented with protease inhibitors (1 mM Pefabloc, 4 μM leupeptin, and 3 μM aprotinin). Thirty micrograms of each cell lysate was denatured (0.5% sodium dodecyl sulfate [SDS], 1% β-mercaptoethanol) at 100°C for 10 min and separated by SDS–10% polyacrylamide gel electrophoresis (SDS–10% PAGE). After transfer, the nitrocellulose

membrane was saturated in Tris-buffered saline with 5% milk powder and 0.05% Tween 20 overnight at 4°C. Immunostaining was performed in the same buffer with 1% milk powder. Blots were developed by the use of horseradish peroxidase-conjugated antibodies (Jackson Laboratory) together with an enhanced chemiluminescence kit (Amersham Pharmacia).

Immunoprecipitation analysis. Transfected cells were labeled for 24 h with [^{35}S]cysteine and [^{35}S]methionine (200 μCi per plate; specific activities of 1,075 and 1,175 Ci/mmol, respectively; NEN) and lysed in a buffer (1% Nonidet P-40, 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride. The envelope proteins were immunoprecipitated with the anti-TM MAb, with the anti-p24 MAb used as a control, for 1 h. Immune complexes were precipitated with protein A- and G-Sepharose beads and washed three times with RIPA buffer (0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 50 mM Tris-HCl [pH 7.4], 150 mM NaCl). Immunoprecipitated proteins were eluted for 5 min at 100°C in a buffer containing 100 mM Tris-HCl (pH 6.8), 1% SDS, 10% glycerol, and 5% β-mercaptoethanol and were analyzed by SDS–10% PAGE.

Flow cytometry and cell surface expression. Cells were washed in PBS and harvested by a 10-min incubation at 37°C with 0.02% Versene in PBS. The cells (10^6) were stained for 1 h at 4°C with the anti-TM MAb in PBA (PBS with 2% fetal calf serum and 0.1% sodium azide). The cells were washed once with PBA and then incubated with a fluorescein isothiocyanate-conjugated antibody (FITC-Fluorescence; DAKO). The cells were washed twice with PBA and counterstained with 3 mM propidium iodide. The fluorescence of living cells was analyzed with a fluorescence-activated cell sorter (FACSCalibur; Becton Dickinson).

In vitro and in cell deglycosylation treatments. (i) PNGase F digestion. Thirty micrograms of whole protein extract was denatured at 100°C for 10 min. The sample was digested with 500 U of peptide N-glycosidase F (PNGase F) in a buffer containing 5 mM sodium phosphate, pH 7.5, and 1% NP-40. Each reaction was performed in 100 μl at 37°C for 4 h. The samples were analyzed by SDS–10% PAGE, and the blot was probed with the anti-TM-EnvW MAb.

(ii) Tunicamycin treatment. Cells were treated with 100 mg of tunicamycin (Calbiochem)/ml for 20 h at 37°C before protein extraction. Thirty micrograms of each cell lysate was separated by SDS–10% PAGE, and the blot was probed with the anti-SU-EnvW MAb.

***Trans*-dominant-negative interference assay.** TELCeB6 cells were transfected with 0.5 μg of the wild-type HERV-W plasmid and increasing amounts of the AAAR mutant plasmid (0, 0.02, 0.1, 0.5, 1, 1.5, and 2 μg). The DNA mixture was supplemented with a DNA carrier (up to 2.5 μg). Thirty micrograms of each cell lysate was separated by SDS–10% PAGE. The blot was probed with the anti-TM-EnvW MAb, and samples were standardized with an anti-actin MAb.

Cell-cell fusion assay. Transfected TELCeB6 cells were overlaid with HeLa indicator cells. Cocultures were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) for 4 h at 37°C to reveal β-galactosidase activity and with May-Grünwald and Giemsa solutions (Sigma) to visualize the nuclei of the producer cells according to the manufacturer's recommendations. Determinations of the fusion activities of the transfected envelope glycoproteins were performed after 36 h of coculture. The fusion index was defined as follows: $(N - S)/T$, where N is the number of nuclei in syncytia, S is the number of syncytia, and T is the total number of nuclei counted. The results are expressed as percentages of the fusion indices.

RESULTS AND DISCUSSION

Env ERVWE1 is synthesized as a gPr73 precursor and cleaved into gp50 SU and gp24 TM subunits. We studied Env ERVWE1 constitutive expression by using the human placental trophoblast BeWo model. Two polyclonal antibodies, raised against Env ERVWE1 SU (anti-SU-EnvW) and Env ERVWE1 TM (anti-TM-EnvW), allowed the detection of a 73-kDa Env ERVWE1 precursor (gPr73) under reducing conditions (Fig. 1B). This precursor was processed into two subunits, defined as a 50-kDa SU domain (gp50) and a 24-kDa TM domain (gp24). The Env ERVWE1 N-glycosylation pattern was studied by PNGase F digestion of the mature protein. This treatment reduced the Env ERVWE1 precursor's molecular mass approximately 20 kDa (Fig. 1B). Assuming that each oligosaccharide contributes about 2.8 kDa to the molecular mass of the protein (55), this shift is consistent with the seven predicted sites. Indeed, the molecular masses of SU and TM shifted about 18 and 3 kDa, respectively. The single TM N-glycosylation site is a feature of the D-type interference group of retroviral envelope glycoproteins, such as the betaretrovirus Mason-Pfizer monkey virus (M-PMV) or gammaretrovirus baboon endogenous retrovirus (BaEV) envelope glycoproteins (35). Altogether, these results indicate that the overall maturation process of syncytin in a human placental trophoblast model is similar to the maturation of infectious retrovirus envelopes (12).

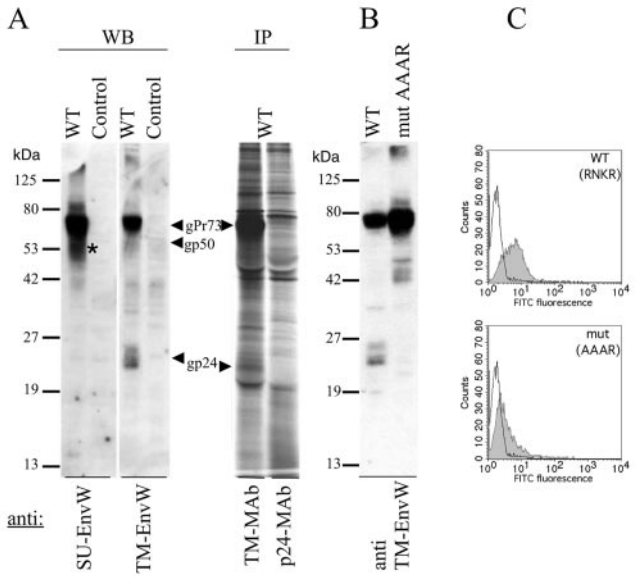

FIG. 2. Env ERVWE1 expression in transfected human cells. (A) For Western blot analysis (WB), cells transfected with wild-type HERV-W (WT) and antisense orientation (control) plasmids were separated by SDS–10% PAGE. The blots were probed with anti-SU-EnvW and anti-TM-EnvW. For immunoprecipitation analysis (IP), cells expressing the WT envelope protein were immunoprecipitated with the anti-TM MAb and the anti-p24 MAb (control). Immunoprecipitated proteins were analyzed by SDS–10% PAGE. Arrowheads indicate the positions of gPr73, gp50 SU, and gp24. The asterisk indicates the position of the glycosylated SU protein. (B) Furin cleavage site mutant analysis. Cell lysates expressing WT and furin cleavage site mutant (AAAR) envelope proteins were separated by SDS–10% PAGE and probed with anti-TM-EnvW. (C) Furin cleavage site mutant cell surface expression. Cells expressing the WT (RNKR, shaded area), furin cleavage site mutant (AAAR, shaded area), or antisense orientation (control, white area) envelope were stained with the anti-TM MAb. The cells were analyzed by flow cytometry.

In order to molecularly characterize the amino acid sequences involved in the consecutive steps of the maturation process, we designed an experimental cellular model consisting of TELCeB6 cells transiently transfected with *env*-carrying plasmids. Both polyclonal antibodies allowed the detection of the gPr73 precursor (Fig. 2A). The polyclonal anti-TM-EnvW antibody allowed the detection of gp24 species with molecular masses ranging from 23 to 25 kDa, which probably corresponded to processing intermediates. In addition, the gPr73 and gp24 proteins were characterized by an immunoprecipitation assay using a monoclonal anti-TM MAb (Fig. 2A) in parallel with the protein subunit determination in BeWo cells.

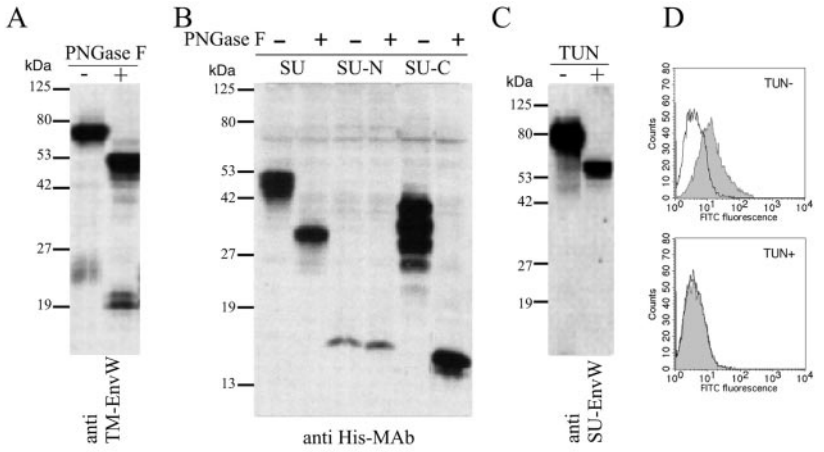

FIG. 3. Analysis of Env ERVWE1 N-linked glycosylation. (A) Env ERVWE1-expressing cell lysates were digested with PNGase F (+) or left untreated (−) and then analyzed by SDS–10% PAGE. The blot was probed with anti-TM-EnvW. (B) N-glycosylation pattern of the SU subunit. Cells transfected with the SU, SU-N, and SU-C truncated envelope proteins were treated (+) or not (−) with PNGase F. The samples were separated by SDS–10% PAGE, and the blot was probed with an anti-His MAb. (C) Env ERVWE1-expressing cell lysates were treated with tunicamycin (+) or left untreated (−) and then separated by SDS–10% PAGE. The blot was probed with anti-SU-EnvW. (D) Env ERVWE1 cell surface expression of treated (TUN+, shaded area) and untreated (TUN−, shaded area) cells. Antisense orientation envelope-expressing cells were used as a control (white area). The cells were stained with the anti-TM MAb and analyzed by flow cytometry.

The diffuse band detected by the polyclonal anti-SU-EnvW MAb may represent the monomeric gp50 SU domain. The aspect of the band may have resulted from a heavier and more heterogeneous glycosylation in these cells than in BeWo cells. However, due to (i) their high level of expression of the precursor protein and (ii) their previous usage to demonstrate the fusogenic property (7), TELCeB6 cells transiently transfected with *env*-based plasmids have been conserved as a model for studying the maturation steps of syncytin.

To confirm that there is a specific gPr73 proteolytic cleavage on the consensus furin cleavage site RNKR, we designed a mutant in which the amino acid sequence was replaced with an AAAR sequence. No gp24 was detected in AAAR mutant-transfected cells (Fig. 2B), demonstrating the absence of cleavage. A sensitive cell-cell fusion assay showed that the AAAR mutant was not able to promote syncytium formation, indicating the absence of a functional envelope at the cell membrane (data not shown). Nevertheless, immunolabeling with the anti-TM MAb induced a fluorescence intensity shift in the AAAR mutant-expressing cells (Fig. 2C), demonstrating that this mutant protein can reach the cell surface. This mutant exhibited delayed kinetics of appearance on the membrane compared to the wild-type envelope. All of these features leading to the nonfusogenic phenotype were strictly conserved in a second furin cleavage site mutant bearing a single amino acid mutation at position four, giving RNKT (data not shown). This R-to-T mutation was indeed previously shown to eliminate the cleavage and fusogenicity of HIV-1 and human foamy virus envelopes, although both mutated glycoproteins were detected at the cell membrane (2, 14). Altogether, these results show that the gPr73 proteolytic cleavage is specific for the consensus furin cleavage site and required for the acquisition of fusogenicity.

Env ERVWE1 is a moderately glycosylated protein. PNGase F digestion of the Env ERVWE1 mature protein and a tunicamycin treatment of Env ERVWE1-expressing cells confirmed the number of observed N-glycosylation sites on native proteins in the human placental trophoblast model. The unglycosylated Env ERVWE1 molecule exhibited a 53-kDa molecular mass, corresponding to gPr73 lacking seven N-glycans (Fig. 3A). The unglycosylated TM subunit had a 20-kDa molecular mass, corresponding to gp24 lacking a unique site (Fig. 3A). PNGase F digestion analysis of SU, SU-N, and SU-C deletion mutants suggested that these glycosylation sites correspond to the ones predicted by sequence analysis. The SU protein showed a relatively heterogenous glycosylation pattern ranging from 46 to 39 kDa (Fig. 3B). PNGase F treatment induced a single 29-kDa band. This molecular size reduction was consistent with the presence of the six expected N-glycosylation sites. The SU-C protein showed a complex pattern consisting of 36-, 31-, 27.5-, and 24-kDa bands that may represent various glycosylation statuses (Fig. 3B). Nevertheless, as observed for the SU subunit, the deglycosylation treatment produced a single 15-kDa band. This result was consistent with the predicted localization of the six N-glycosylation sites in the carboxy-terminal part of SU, as observed for other retroviral SU domains (17, 18). This point was confirmed by the absence of any deglycosylation-induced molecular weight variation within the SU-N construct.

env-transfected cells were treated with tunicamycin, a blocking agent of the nascent protein during the early events of the maturation process which prevents the transfer of saccharidic units to the peptide chain. Western blot analysis showed that tunicamycin treatment induced a similar deglycosylation pattern to that observed with PNGase F treatment (Fig. 3C). Furthermore, a flow cytometry analysis suggested an absence of Env ERVWE1 expression at the cell surface after tunicamycin treatment (Fig. 3D). The absence of syncytium formation in cell-cell fusion assays with tunicamycin-treated cells confirmed the absence of functional protein at the cell surface (data not shown). Altogether, these results suggest that tuni-

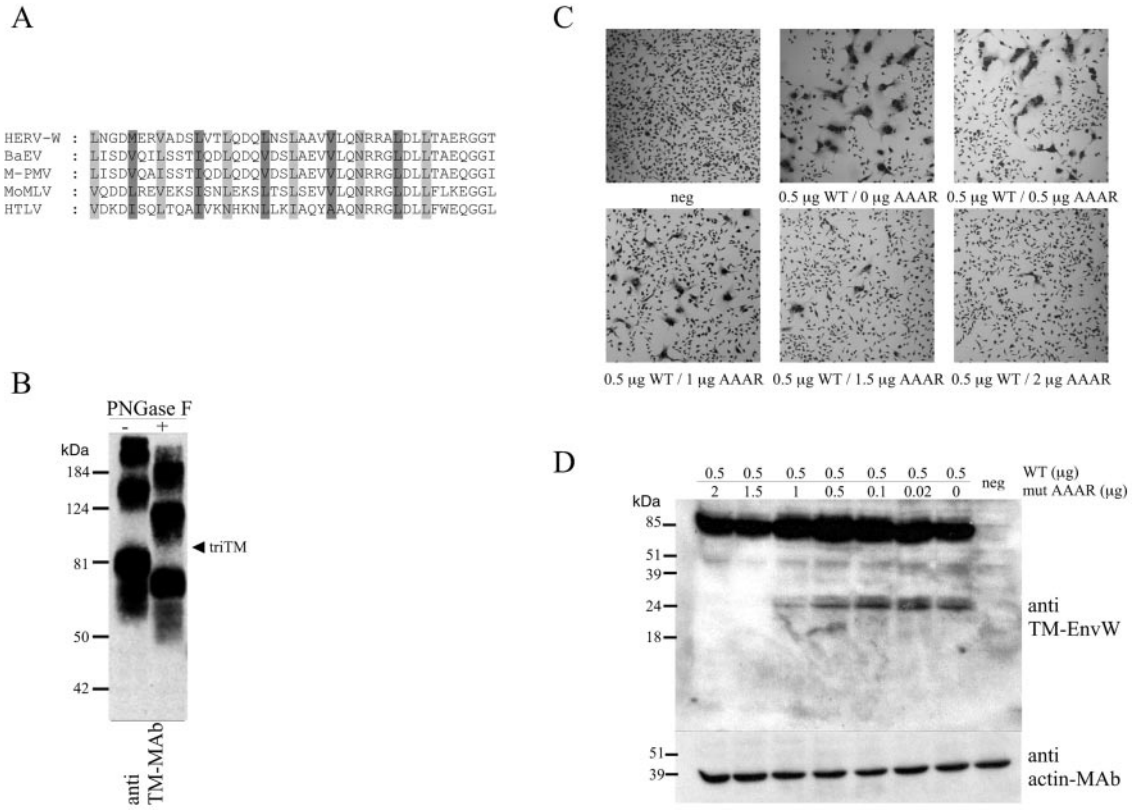

FIG. 4. Env ERVWE1 oligomerization. (A) Sequence alignment of leucine zipper-like motifs in retroviral transmembrane subunits. A comparison of predicted D-type (BaEV and M-PMV) and characterized C-type (Moloney MLV [MoMLV] and HTLV) sequences is shown. "a" and "d" residues of the heptad repeat, highlighted in dark gray and light gray, respectively, organize on the same side of the α helix. Leucine residues at position "a" are nonexclusively conserved and are frequently replaced with valine residues. Residues found at position "d" are nonpolar and hydrophobic residues. The Env ERVWE1 leucine zipper-like motif starts at position 346. (B) Env ERVWE1 oligomerization. Env ERVWE1-expressing cell lysates were treated (+) or not (−) with PNGase F and separated by SDS–10% PAGE under nonreducing conditions. The blot was probed with the anti-TM MAb. The arrowhead indicates oligomeric complexes composed of three TM subunits (triTM). (C) Effect of dominant-negative mutant on syncytium formation. Transfected cells were overlaid with HeLa indicator cells. Magnification, ×40. (D) Effect of dominant-negative mutant on Env ERVWE1 processing. Cells were transfected with 0.5 μg of the WT plasmid and increasing amounts of the AAAR mutant plasmid (0, 0.02, 0.1, 0.5, 1, 1.5, and 2 μg). Cell lysates were separated by SDS–10% PAGE. The blot was probed with anti-TM-EnvW and standardized with an anti-actin MAb.

camycin-related inhibition of the early maturation step leads to misfolded proteins that remain trapped in the ER (36).

The high-mannose oligosaccharide composition of several viral envelope glycoproteins confers on them the ability to bind C-type lectin surface molecules such as DC-SIGN (dendritic cell-specific ICAM-grabbing nonintegrin) and L-SIGN (liver/lymph-node-specific ICAM-3-grabbing nonintegrin, or DC-SIGNR). Class I fusion proteins of *Lentivirus* (3, 11, 38) and *Filovirus* (30) and class II *Hepacivirus* (39) envelope proteins have also been reported to display this property. However, this observation does not extend to all class I fusion proteins, as demonstrated by the gammaretrovirus MLV (16, 26). We tested whether Env ERVWE1 contained a high-mannose glycan composition via its ability to bind DC-SIGN. We designed a DC-SIGN-specific enzyme-linked immunosorbent assay consisting of DC-SIGN-expressing HeLa cells as the capture phase and specific antibodies directed against the SU, SU-N, and SU-C recombinant proteins as the detection system. The supernatants of all three *env*-transfected TELCeB6 cell types were incubated with DC-SIGN-expressing HeLa cells, and the ability of each construct to bind DC-SIGN was determined by use of the specific antibodies (see http://www.ens-lyon.fr/CNRS-bioMerieux/Retrovirology/supJVIcheynet). Both the SU and SU-C proteins bound to DC-SIGN, although with different efficiencies, probably due to structural differences. As expected, the unglycosylated SU-N domain was unable to bind DC-SIGN.

These results show that the HERV-W envelope is a moderately glycosylated protein, as observed for the related gamma- and betaretrovirus envelopes. Conversely, HERV-W glycans, which are essential for proper folding and function, consist mainly of high-mannose N-glycans located at the carboxy terminus of the SU domain.

Env ERVWE1 organizes as homotrimers. The gp24 transmembrane subunit contains two broadly conserved retroviral sequences, the leucine zipper-like motif (Fig. 4A) and the CX_6CC motif (Fig. 5A), which suggests that the envelope precursor can oligomerize and that SU and TM subunits are covalently linked. The oligomerization status of both the envelope precursor and the TM subunit was analyzed by immu-

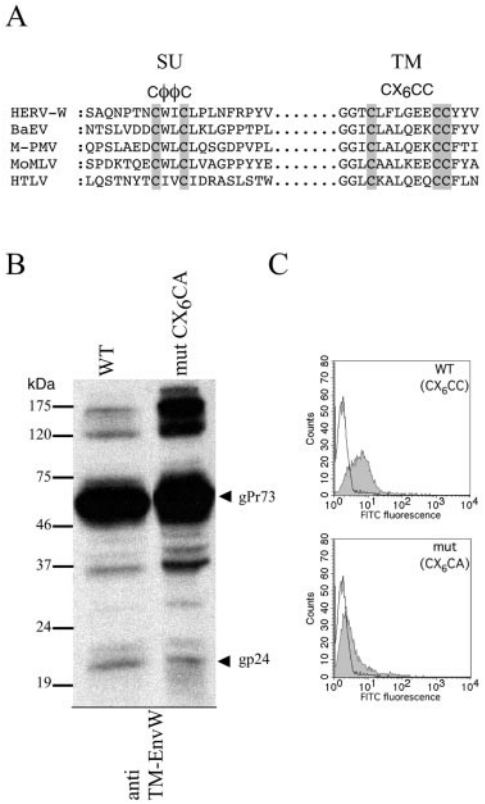

FIG. 5. Env ERVWE1 conserved CX_6CC motif. (A) Sequence alignment of CϕϕC (SU) and CX_6CC (TM) motifs. A comparison is shown with D-type (BaEV and M-PMV) and C-type (MoMLV and HTLV) sequences. Gray boxes correspond to conserved cysteine residues. (B) CX_6CA mutant expression. Cells lysates expressing WT and CX_6CA mutant envelope proteins were separated by SDS–10% PAGE and probed with anti-TM-EnvW. Arrowheads indicate the positions of gPr73 and gp24. (C) CX_6CA mutant cell surface expression. Cells expressing the WT (CX_6CC, shaded area), mutant (CX_6CA, shaded area), or antisense orientation (control, white area) envelope were stained with the anti-TM MAb and analyzed by flow cytometry.

noblotting under nonreducing conditions (Fig. 4B). The monoclonal anti-TM MAb allowed the detection of several bands, of about 80, 140, and 200 kDa. After PNGase F treatment, the 80-kDa band decreased about 10 kDa. Considering its molecular size and the presence of a unique N-glycan site in gp24, we identified this band as an oligomeric complex composed of three TM subunits. Higher bands in the gel seemed to be dimers and trimers of the Env ERVWE1 precursor, although the gel resolution did not permit us to make definite conclusions. Therefore, our results indicate that the Env ERVWE1 glycoprotein is able to form homotrimers.

To understand whether this trimerization is necessary for efficient Env ERVWE1 maturation, we developed a *trans*-dominant-negative interference assay by using the furin cleavage site AAAR mutant. A constant amount of wild-type envelope was coexpressed with increasing amounts of the defective AAAR mutant. The cell-cell fusion assay showed that an increase in the AAAR mutant resulted in a simultaneous decrease in syncytium formation (Fig. 4C). More precisely, a 3-fold excess of AAAR mutant caused a substantial 10-fold decrease in syncytium formation. This observation led us to suggest that nonfunctional heterotrimers reach the cell membrane. As expected, fluorescence-activated cell sorting analysis showed the presence of Env proteins at the cell surface (data not shown), although we were unable to discriminate between AAAR mutant and wild-type proteins. Western blot analysis showed that an increase in the amount of AAAR mutant correlated with a decrease in gp24 (Fig. 4D). We hypothesize that the associated mutant may alter the cleavage of the native envelope molecules, contributing to the reduction in syncytium formation. Thus, the *trans*-dominant-negative interference assay confirmed that Env ERVWE1 requires an accurate oligomeric structure to undergo the processing pathway leading to fusogenicity.

The Env ERVWE1 sequence contains a typical disulfide isomerase motif, CϕϕC, in the SU domain and a CX_6CC motif in the TM domain (Fig. 5A). MLV TM crystallography studies showed that the first two cysteines of the CX_6CC motif can form a stable disulfide bond (13), leaving the third cysteine free to form a disulfide bond with the CϕϕC motif. We chose to introduce a neutral substitution, CX_6CA, in order to avoid alterations of the structure and to analyze the effect on the maturation process and the envelope's function. This mutation did not appear to affect the level of expression or the cleavage of the precursor protein (Fig. 5B). However, no syncytia were observed for the CX_6CA mutant in the sensitive cell-cell fusion assay (data not shown), indicating that the third cysteine is essential for the fusogenic phenotype. Flow cytometry analysis showed that the CX_6CA mutant was expressed at the cell surface (Fig. 5C), although with a lower apparent efficiency than that of the wild-type envelope. The absence of fusion by the CX_6CA mutant may have been due to (i) a huge shedding of gp50 or (ii) an alteration of the sequential events leading to the fusogenic state of the homotrimer, e.g., an altered SU-TM communication or interaction. The absence of detectable soluble SU in cell culture supernatants seems to exclude shedding as the major cause of the CX_6CA mutant's altered fusion (data not shown). Thus, the CX_6CC carboxyl cysteine mutation probably contributed to incorrect folding of the mutant envelope, as previously described for cysteine substitution in the CϕϕC and CX_6CC motifs of gamma- and betaretrovirus envelopes (20, 46).

The Env ERVWE1 intracytoplasmic tail is required for fusion. The retroviral protease cleavage site and R peptide region are missing from the Env ERVWE1 69-amino-acid intracytoplasmic tail (7) without altering the constitutive fusion competence of Env ERVWE1. Moreover, the human genome does not contain any HERV-W *pro* genes in a favorable translational context (34, 49). D-type and C-type mammalian retrovirus R peptides exert a fusion inhibition effect (53, 54). R peptide cleavage by the retroviral protease allows the envelope protein to be fusion competent (9, 40, 41). These characteristics led us to question the role of the intracytoplasmic tail in the fusion process. Hence, we designed a tailless mutant (the CYT-1 mutant) (Fig. 6A). The removal of the intracytoplasmic tail dramatically reduced the envelope protein fusogenicity in the cell-cell fusion assay, as visualized by a decrease in the fusion index. Next, we focused on localizing the fusion determinants more precisely in the intracytoplasmic tail. We generated truncation mutants (the CYT-16 and CYT-31 mutants) by

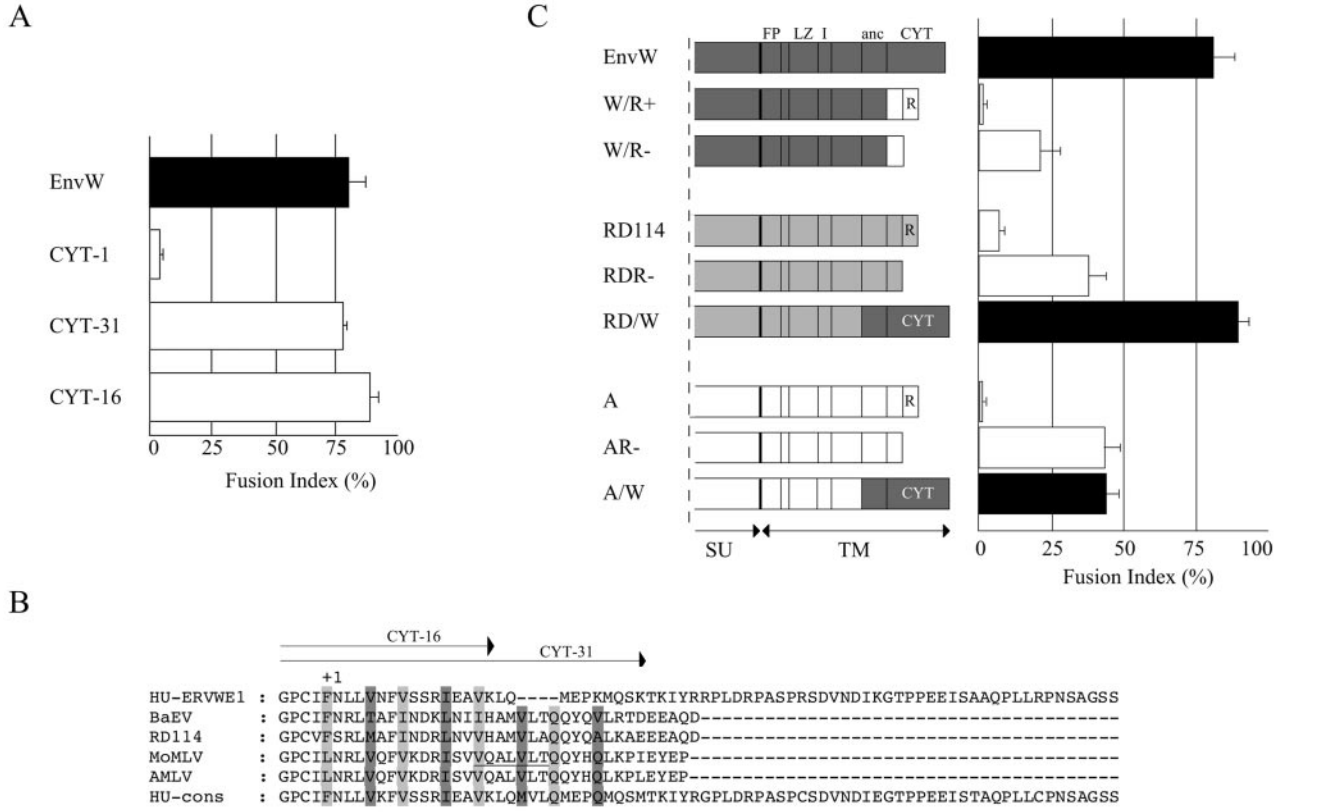

FIG. 6. Involvement of Env ERVWE1 intracytoplasmic tail in fusogenic activity. (A) Localization of fusion determinants. Intracytoplasmic tail mutant envelope glycoproteins (CYT-1, CYT-16, and CYT-31) were tested in a cell-cell fusion assay. The results are expressed as percentages of the fusion indices (means ± standard deviations; $n = 5$). (B) Sequence alignment of intracytoplasmic tails of ERVWE1 (HU-ERVWE1), BaEV, RD114, MoMLV, and amphotropic MLV (AMLV) and the human *env* HERV-W consensus sequence (HU-cons). +1, first residue of the intracytoplasmic tail. "a" residues of the conserved helical structure (heptad repeat) are highlighted in dark gray, and "d" residues are highlighted in light gray. Arrows represent the intracytoplasmic lengths of the CYT-16 and CYT-31 truncation mutants. A viral protease-specific site is underlined in the MoMLV sequence. (C) Role of conserved domain of Env ERVWE1 intracytoplasmic tail. The RD114, RDR$^-$, A, and AR$^-$ envelope glycoproteins and the chimeric envelope glycoproteins W/R$^+$, W/R$^-$, RD/W, and A/W (see Material and Methods) were tested in cell-cell fusion assays. The results are expressed as percentages of the fusion indices (means ± standard deviations; $n = 5$).

eliminating the carboxy-terminal residues (Fig. 6B). As illustrated in Fig. 6A, the deletion of half (CYT-31 mutant) or up to three quarters (CYT-16 mutant) of the intracytoplasmic tail did not affect the fusogenicity of the mutants. Therefore, no critical fusion determinants are located in the carboxy-terminal part of the protein. This observation suggested that the Env ERVWE1 fusion determinants reside in the first 16 residues of the intracytoplasmic tail. Sequence alignments of the first 16 Env ERVWE1 residues (Fig. 6B) revealed the local conservation of a predicted helical structure which is presumably involved in cell-cell fusion (45). Note that the CYT-16 fusogenic mutant containing the shorter tail induced the formation of syncytia containing up to 160 nuclei, compared to 100 nuclei for the wild-type envelope. An overall similar situation was previously described for the HTLV envelope protein, which did not present any defined R-related cleavage site and contained fusion determinants in the first eight residues of the intracytoplasmic tail (24). To precisely determine the role of this region in Env ERVWE1's constitutive fusogenic activity, we developed swapping chimeras between Env ERVWE1 and feline endogenous retrovirus (RD114) and amphotropic MLV (A) envelope proteins. In a first approach, we tested whether the Env ERVWE1 fusogenic activity could be controlled by an infectious retrovirus R peptide region. The Env ERVWE1 intracytoplasmic tail was replaced with the complete (W/R$^+$ mutant) or a shortened R-less version (W/R$^-$ mutant) of the amphotropic MLV intracytoplasmic tail. As illustrated in Fig. 6C, the presence of the amphotropic R peptide region restored fusion inhibition control to the W/R$^+$ mutant. The fusogenicity of this chimera could be restored by the R peptide truncation (W/R$^-$ mutant). This observation is in agreement with previously described results in which the fusogenic activities of wild-type gamma- and betaretrovirus envelope proteins were restored by the introduction of a stop codon after the viral protease cleavage site (9, 40, 41). In a second approach, we questioned the role of the Env ERVWE1 intracytoplasmic tail in the cell-cell fusion regulation of infectious prototypes (Fig. 6C). As expected, the introduction of a stop codon to wild-type RD114 (RDR$^-$) and A (AR$^-$) restored the cell-cell fusion activities of these envelope proteins. The replacement of their intracytoplasmic tails with the Env ERVWE1 intracytoplasmic tail (RD/W and A/W mutants) conferred on them a constitutive fusogenicity as high as the wild-type Env ERVWE1 activity in the case of the RD/W chimera. Altogether, these results

confirm the role of the Env ERVWE1 conserved region in the fusion process. We suggest that the sequence from the 16th residue on may be a remnant of the R peptide region that has lost its fusion inhibition control of Env ERVWE1 fusogenicity. However, we cannot exclude the possibility that the Env ERVWE1 intracytoplasmic tail has evolved to bypass the viral cleavage requirement or has adapted to cellular protease cleavage. To date, with the various approaches developed to test this hypothesis, we cannot confirm the existence of either a cleaved Env ERVWE1 or a cellular protease-defective cell line.

Conclusion. The results presented here provide evidence that the syncytin/HERV-W envelope glycoprotein expressed in placental trophoblastic BeWo cells is synthesized as a gPr73 precursor which is specifically cleaved at a consensus furin cleavage site to give rise to two mature subunits, gp50 SU and gp24 TM. The mature envelope protein possesses seven N-glycosylation sites, including one in the TM subunit which is essential for correct envelope protein folding and function. The carboxy-terminal domain of the SU subunit is mainly composed of high-mannose N-glycans, as shown by its interaction with DC-SIGN. The significance of the Env ERVWE1–DC-SIGN interaction deserves further investigation. Thus, both proteins may theoretically colocalize, as suggested by the villous (15, 28) and extravillous (31, 43) trophoblast expression of syncytin versus the chorionic villus fetal capillary and maternal decidua expression of DC/LC-SIGN molecules (44). Such an interaction would suggest that Env ERVWE1 exhibits other physiological properties besides fusogenicity, e.g., an immunological function. In addition, the observation that Env ERVWE1 is associated with detergent-resistant membranes (see http://www.ens-lyon.fr/CNRS-bioMerieux/Retrovirology/supJVIcheynet) should be scrutinized with regard to the risk of HIV pseudotyping by syncytin (1, 27).

For its fusion competence, Env ERVWE1 is organized as a homotrimeric structure. The SU-TM interaction is probably stabilized by a covalent bond between the SU C$\phi\phi$C motif and the TM CX_6CC motif. Finally, fusion competence does not apparently rely on an R-like peptide cleavage but on the first 16 residues of the intracytoplasmic tail. Constitutive fusogenicity may result from the remnant of an R peptide region that has lost its fusion inhibition control. This hypothesis is reinforced by studies of the selective pressure exerted on the *env ERVWE1* sequence (8). In fact, among all *env ERVWE1* sequences found in the human genome, only the ERVWE1 locus coding sequence shows a 12-bp (four-amino-acid) deletion in the intracytoplasmic tail, located after the first 16 conserved residues. This deletion modifies the potential retroviral protease cleavage site and presumably disturbs the end of the helical structure (Fig. 6B). The insertion of the coding sequence for these four amino acids into the Env ERVWE1 plasmid inhibited the envelope glycoprotein fusogenic activity without altering its maturation process. This observation supports the hypothesis of an infectious ancestor with an original R peptide fusion inhibition function (8).

We recently demonstrated that the fusogenic activity of Env ERVWE1/syncytin is preserved in human polymorphic variants and in orthologous loci of chimpanzees, gorillas, orangutans, and gibbons (32). The conservation of all of the Env gene features supports the synthesis, assembly, and processing of the envelope glycoprotein as described in this study. Hence, this domesticated retroviral glycoprotein represents an interesting and relatively simple model for further analyses of the mechanisms leading to cell-cell (or possibly virus-cell) membrane fusion.

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